ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2007, Vol. 33, No. 1, pp. 73–82. © Pleiades Publishing, Inc., 2007.
Original Russian Text © A.S. Silchenko, S.A. Avilov, V.I. Kalinin, V.A. Stonik, A.I. Kalinovsky, P.S. Dmitrenok, V.G. Stepanov, 2007, published in Bioorganicheskaya Khimiya, 2007, Vol. 33, No. 1, pp. 81–90.

STRUCTURAL STUDIES

Monosulfated Triterpene Glycosides from *Cucumaria okhotensis* Levin Et Stepanov, a New Species of Sea Cucumbers from Sea of Okhotsk

A. S. Silchenko[a], S. A. Avilov[a], V. I. Kalinin[a, 1], V. A. Stonik[a], A. I. Kalinovsky[a], P. S. Dmitrenok[a], and V. G. Stepanov[b]

[a] *Pacific Institute of Bioorganic Chemistry, Far-East Division, Russian Academy of Sciences, pr. 100-letya Vladivostoka 159, Vladivostok, 690022 Russia*

[b] *Pacific Institute of Geography (Kamchatka Branch), Far-East Division, Russian Academy of Sciences, ul. Partizanskaya 6, Petropavlovsk-Kamchatski, 683000 Russia*

Received April 7, 2006; in final form, April 10, 2006

Abstract—Three compounds were isolated from the fraction of monosulfated triterpene glycosides from *Cucumaria okhotensis*, a new sea cucumber species, and their structures were elucidated. First of them, okhotoside A_1-1, is a new glycoside containing tetrasaccharide sugar moiety; the second, okhotoside A_2-1, is a new pentaoside with a glucose residue in the second position of sugar moiety (such a structural peculiarity has been found in holothurians of the genus *Cucumaria* for the first time); and the third is a previously known pentaoside cucumarioside A_0-1. The species-specificity of the triterpene glycosides from *C. okhotensis* was revealed, which justifies the description of this sea cucumber as a new species.

Key words: chemotaxonomic markers, Cucumaria okhotensis, triterpene glycosides

DOI: 10.1134/S1068162007010098

INTRODUCTION

Holothurians, or sea cucumbers, are the representatives of one of five nowadays living classes of the sea organisms belonging to the type echinoderms (Echinodermata).[2] Some holothurian species, including those belonging to the genus *Cucumaria*, are subjects of a trade. Moreover, the curative (medical) properties of products from holothurians are highly esteemed in Asia. It has now been established that the biological action of holothurian extracts is in many respects due to the presence in them of triterpenoid glycosides, low-molecular bioregulators possessing a wide spectrum of physiological activities [1].

The structural study of the triterpenoid glycosides of the holothurians belonging to the genus *Cucumaria* has been started in 1980s [2, 3], and, for this time, the glycoside composition of seven species of this genus have been investigated [4]. For example, the study of glycoside composition of such mass production species of holothurians as Far-Eastern *Cucumaria japonica* and North Atlantic *C. frondosa* [5] have shown that each of them has a specific set of triterpenoid glycosides. The use of the obtained information helped to solve the problem of their taxonomy [6].

The *C. japonica* was considered until recently to be distributed from Bering sea up to southern islands of Japan. However, several new species of the genus have been described for the last years: *C. djakonovi*, *C. savelijevae* [7], *C. conicospermium* [8], *C. levini* [9], and also *C. okhotensis* [10]. The new data on the systematics of Far-Eastern cucumaria allowed the conclusion that the species *C. japonica* is assembling (because all the recently described holothurians have been classified as *C. japonica*), and, actually, the areal of its distribution is significantly narrower than was earlier considered.

The difficulty of species definition of the *Cucumaria* holothurians is determined to a high degree by the variability of the ossicle form, which is considered to be a basic characteristic of the holothurian species attribution. This situation makes obvious the value and prospects of the use of structural analysis of triterpenoid glycosides to solve the controversial questions of tax-

[1] Corresponding author; phone: (4232) 311-168; fax: (4232) 31-4050; e-mail: kalinin@piboc.dvo.ru.

[2] Abbreviations: MALDI TOF MS, Matrix-Assisted Laser Desorbtion/Ionization Time of Flight Mass Spectrometry and NMR special techniques: DEPT, Distortionless Enchancement by Polarization Transfer; HSQC, Heteronuclear Single Quantum Connectivity, HMBC; Heteronuclear Multiple Bond Connectivity; COSY, Correlation Spectroscopy; NOESY, Nuclear Overhauser and Exchange Spectroscopy; and TOCSY, Total Correlation spectroscopy.

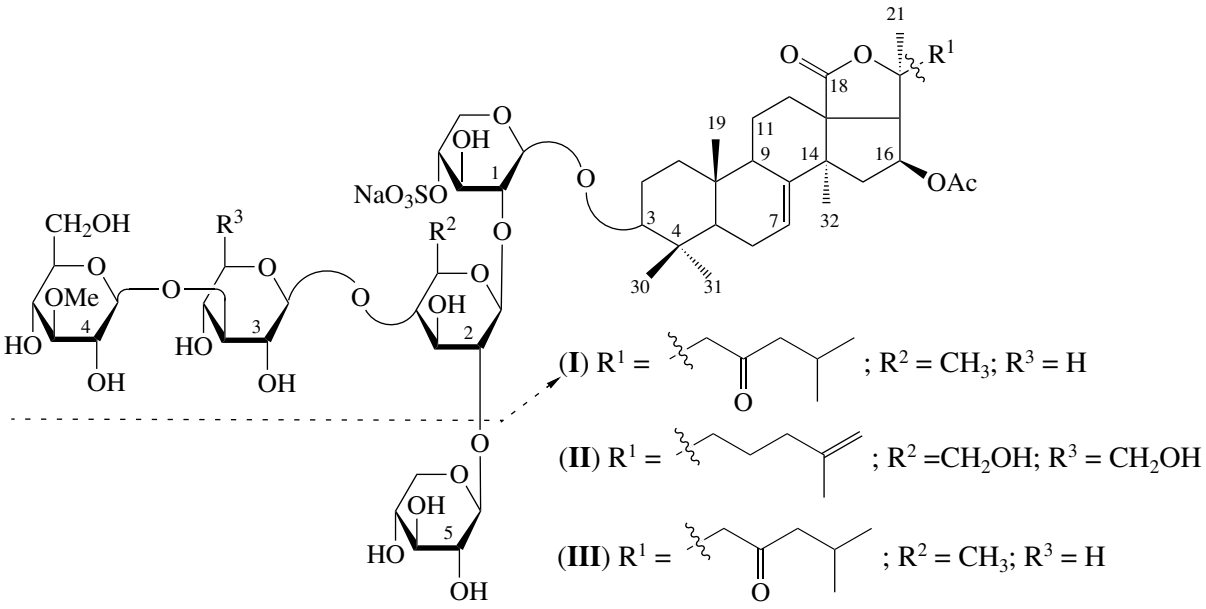
onomy. For example, the study of triterpenoid glycosides of *C. conicospermium* have revealed their significant structural differences from the glycosides of *C. japonica* and have confirmed the validity of the isolation of this holothurian in a new species [11].

Another important aspect of studying the glycoside composition of the production species of holothurians is the possibility of applied use of the obtained information. The accumulated data on the structures of triterpenoid glycosides and on their biological activities allowed the researchers to begin the development of medical preparations and biologically active food additives on the basis of glycoside fractions of holothurians *C. japonica* [12, 13] and *C. frondosa* [14]. In connection with the description of new Far-Eastern species of holothurians of the genus *Cucumaria* (earlier all the produced in large scale Far-Eastern cucumariids were considered as belonging to one species, *C. japonica*), it is topical to standardize the raw material at manufacturing medicinal and veterinary preparations and also to reveal an opportunity of using different species of the genus *Cucumaria* as a source of glycosides for the development of new medicinal products.

The purpose of this work is the study of glycosides of the holothurian *C. okhotensis* for the confirmation of its species isolation and finding of an opportunity of using *C. okhotensis* as a source of glycosides for new medical products.

RESULTS AND DISCUSSION

The glycoside fraction of the holothurian *C. okhotensis* was collected at the western coast of Kamchatka. We isolated from it several glycoside subfractions of various polarities: mono-, di-, and trisulfated compounds also characteristic of other studied species of the genus *Cucumaria* [4, 11]. In this work, we investigated the subfraction of monosulfated glycosides. Three compounds were isolated: two new glycosides, okhotoside A_1-1 (**I**) with a tetrasaccharide linear carbohydrate chain and okhotoside A_2-1 (**II**) with a new pentasaccharide branched carbohydrate chain; the known cucumarioside A_0-1 (**III**) was also identified.



An alcoholic extract of the holothurian *C. okhotensis* was desalted on a Polychrom-1 column and then subjected to repeated column chromatography on silica gel. The subfractions of mono-, di-, and trisulfated glycosides resulted. The subfraction of monosulfated glycosides was separated into individual components by the reversed-phase HPLC. The structures of the isolated compounds were established by an extensive NMR analysis and mass spectrometry and also with the help of chemical transformations.

A comparison of a part of a ^{13}C NMR spectrum of okhotoside A_1-1 (**I**) related to its carbohydrate moiety with the corresponding part of the spectrum of frondoside A_1, isolated earlier from *C. frondosa* [15], showed practically full coincidence of their signals. This allowed us to conclude that the carbohydrate chains of these compounds are identical, including the presence of *O*-sulfate in position 4 of the first residue of xylose (connected with aglycone). In fact, two double signals at δ 104.96 and 105.26 ppm were observed in the ^{13}C NMR spectrum of okhotoside A_1-1 (**I**) (Table 1), which correlated with the signals of four anomeric protons at 4.75 (d, *J* 7.1 Hz), 5.09, 4.86 (d, *J* 7.7 Hz), and 5.35 ppm (d, *J* 7.8 Hz) with the help of a HSQC spectrum. This fact confirms the presence of four monosaccharide residues in the carbohydrate chain of compound (**I**).

Table 1. NMR spectra of the carbohydrate chain of okhotoside A_1-1 (**I**)* in C_5D_5N

Residue	Atom	δ_C, DEPT	δ_H (multiplicity, *J*, Hz)	HMBC	NOESY
Xyl (1)	C^1-1	104.96	4.75 (d, 7.1)	**C**: 3	**H**: 3, 31, H^1-3, H^1-5
	C^1-2	83.25	4.03 (m)	C^1-3, C^2-1	H^2-1
	C^1-3	75.56	4.34 (t, 9.2)	C^1-2, C^1-4	H^1-1, H^1-5
	C^1-4	75.56	5.17 (dt, 9.0, 5.1)		
	C^1-5	64.21	4.81 (dd, 5.3, 11.6)	C^1-4	
			3.77 (m)	C^1-4	H^1-1, ç^1-3
Qui (2)	C^2-1	105.26	5.09**		
	C^2-2	76.17	4.00 (m)	C^2-3, C^2-4	
	C^2-3	75.18	4.07 (m)	C^2-2	H^2-1
	C^2-4	85.78	3.63 (t, 8.9)	C^3-1, C^2-3, C^2-5	H^3-1, H^2-6
	C^2-5	71.60	3.74 (m)		H^2-1
	C^2-6	17.82	1.73 (d, 5.9)	C^2-4, C^2-5	H^3-1
Xyl (3)	C^3-1	104.96	4.86 (d, 7.7)	C^2-4	H^2-4, H^3-3, H^2-6
	C^3-2	73.44	4.01 (m)	C^3-3	
	C^3-3	87.14	4.19 (t, 8.7)	C^4-1, C^3-4, C^3-2	H^3-1
	C^3-4	68.86	4.08 (m)		
	C^3-5	66.39	3.67 (t, 11.0), 4.23 (m)		
3MeGlc (4)	C^4-1	105.26	5.35 (d, 7.8)	C^3-3	H^3-3, H^4-3, H^4-5
	C^4-2	74.89	4.02 (m)		
	C^4-3	87.83	3.72 (t, 8.9)	OMe, C^4-2, C^4-4	H^4-1
	C^4-4	70.50	4.12 (m)		
	C^4-5	78.08	3.98 (m)		
	C^4-6	61.99	4.26 (dd, 5.5, 11.5)		
			4.49 (dd, 2.3, 11.3)		
	OMe	60.60	3.87 (s)		

* The upper index in the designation of atoms corresponds to the number of monosaccharide residue in carbohydrate chain, the figure after the hyphen, to the number of atom in monosaccharide the residue.

** The area is overlapped the by signals of solvent.

The values spin-coupling constants of anomeric protons proves the β-configuration of glycoside linkages [16]. Moreover, a characteristic signal of *O*-methyl group (δ_C 60.60 ppm, δ_H 3.87 ppm) was observed in ^{13}C and ^1H NMR spectra of okhotoside A_1-1, which confirmed the presence of the residue of 3-*O*-methylglucose in the carbohydrate chain of the glycoside. The sequence of monosaccharide linkages in the carbohydrate chain of glycoside (**I**) was established by the analysis of a NOESY-spectrum (Table 1) in which correlations were observed between: aglycone H3 and H1 of the first xylose residue, H2 of the first xylose residue and H1 of the quinovose residue, H4 the quinovose residue and H1 of the second xylose residue, and C1 of the 3-*O*-methylglucose residue and H3 of the second xylose residue. Similar cross peaks were also observed in the HMBC spectrum of okhotoside A_1-1 (**I**) (Table 1).

An acidic hydrolysis of native glycoside (**I**), the subsequent alcoholysis of the monosaccharide mixtures by the action of (*R*)-(–)-2-octanol, acetylation, and the analysis with the help of GLC of independent compounds allowed us to determine the absolute *D*-configuration of all the monosaccharide residues in the carbohydrate chain of okhotoside A_1-1 (**I**): xylose, quinovose, and 3-*O*-methylglucose, present at 2 : 1 : 1 ratio.

Signals of a portion of ^{13}C NMR spectrum of glycoside (**I**) related to aglycone coincided with those of the corresponding portion of spectrum of cucumarioside A_0-1, earlier isolated from *C. japonica* [17, 18]. This fact proved the presence in these compounds of identical aglycones of holostane type with 7(8) double bonds in the polycyclic system and two functional groups: a keto group in the side chain and *O*-acetate group in the aglycone nucleus (Table 2). In fact, the NMR signals in low field of the ^{13}C NMR spectrum of okhotoside A_1-1 (**I**) at δ 145.50 (C8) and 120.31 (C7) ppm indicated the

Table 2. NMR spectra of the aglycone part of okhotoside A_1-1 (**I**) in C_5D_5N

Atom number	δ_C, DEPT	δ_H (multiplicity, *J*, Hz)	HMBC	NOESY
1	35.94	1.44 (m)		
2	26.97	1.91 (m), 2.11 (m)		
3	88.99	3.27 (dd, 3.6, 11.5)		**H**: 1, 5, 31
4	39.43			
5	47.80	1.02 (t, 8.1)		**H**: 3, 31
6	23.20	2.06 (m)		
7	120.31	5.64 (m)		**H**: 15, 32
8	145.50			
9	47.01	3.44 (br. d, 12.3)		**H**: 1, 19
10	35.46			
11	22.38	1.50 (m), 1.79 (m)		
12	30.99	1.98 (m), 2.12 (m)		
13	57.68			
14	47.46			
15	43.57	1.63 (m)		
		2.70 (dd, 8.0, 12.1)	**C**: 13, 14, 17, 32	**H**: 7, 32
16	76.17	5.81 (br. q, 8.7)		**H**: 32
17	55.08	3.25 (d, 9.2)	**C**: 13, 18, 21	**H**: 32, 21, 12
18	179.23			
19	23.85	1.23 (s)	**C**: 1, 5, 9, 10	**H**: 9
20	82.03			
21	29.48	1.59 (s)	**C**: 17, 20, 22	**H**: 17, 22
22	52.66	3.17 (d, 18.3)	**C**: 17, 20, 21, 23	
		3.93 (br. d, 18.3)	**C**: 23	
23	207.67			
24	51.47	2.23 (m)		
		2.38 (dd, 5.9, 16.1)		
25	24.26	2.18 (m)		
26	22.38	0.88 (d, 6.5)	**C**: 24, 25, 27	
27	22.20	0.88 (d, 6.5)	**C**: 24, 25, 26	
30	17.18	1.13 (s)	**C**: 3, 4, 5, 31	
31	28.53	1.29 (s)	**C**: 3, 4, 5, 30	**H**: 3, 5, H1A
32	32.00	1.08 (s)	**C**: 8, 13, 14, 15	**H**: 7, 15, 16, 17
$OCO\underline{C}H_3$	21.11	2.02 (s)		
$O\underline{C}OCH_3$	169.02			

presence of 7(8)-double bond and the signal of carbon atom not bound with protons at δ 207.67 ppm proved the presence of a keto group in position 23. Its position at C23 was proved by the presence of cross peak $(H22)_2$/C23 in the HMBC spectrum and also of signals of an isolated spin system CH_3(27)–CH_3(26)–CH(25)–CH_2(24) in the COSY spectrum. The signal of carbon atom of a carboxyl group at δ 169.02 ppm and also the chemical shift of C16 signal (δ 76.17 ppm) in the spectra of ^{13}C NMR and DEPT proved the presence of acetoxy group at C16, which was confirmed by the presence of cross peak H16/H32 in a NOESY spectrum. The β-configuration of acetoxy group was confirmed by the values of spin-coupling constants.

The empirical formula of okhotoside A_1-1 (**I**) was determined to be $C_{55}H_{85}NaO_{26}S$ by ^{13}C NMR spectrum and the presence of peak of pseudo-molecular ion $[M_{Na} + Na]^+$ at *m/z* 1239 in MALDI TOF MS(+), and also the peak $[M_{Na} - Na]^-$ at *m/z* 1193 in MALDI TOF MS(–). A peak of fragment ion $[M_{Na} + Na - SO_3Na +$

Table 3. NMR spectra of the carbohydrate chain of okhotoside A_2-1 (**II**) in 4 : 1 C_5D_5N–D_2O

Residue	Atom	δ_C, DEPT	δ_H (multiplicity, *J*, Hz)	HMBC	NOESY
Xyl (1)	C^1-1	104.71	4.84 (d, 7.3)	**C**: 3	**H**: 3, H^1-3, H^1-5
	C^1-2	79.64	4.31 (t, 7.9)	C^1-1, C^2-1	H^2-1
	C^1-3	75.55	4.46 (t, 8.9)	C^1-2	H^1-1, H^1-5
	C^1-4	76.08	5.11 (m)	C^1-3	
	C^1-5	64.26	4.91 (dd, 6.1, 11.2)	C^1-1, C^1-3	
			3.90 (t, 11.5)		H^1-1, H^1-3
Glc (2)	C^2-1	101.46	5.52 (d, 7.7)	C^1-2	H^1-2, H^2-3, H^2-5
	C^2-2	82.27	4.09 (t, 8.0)	C^2-1, C^2-3	
	C^2-3	75.61	4.19 (t, 8.9)	C^2-2, C^2-4	H^2-1, H^2-5
	C^2-4	80.55	4.14 (t, 9.1)	C^3-1, C^2-3	
	C^2-5	75.85	3.79 (m)		H^2-1, H^2-3
	C^2-6	61.06	4.47 (m)	C^2-5	
			4.38 (m)		
Glc (3)	C^3-1	103.61	5.08 (d, 8.0)	C^2-4	H^2-4, H^3-3, H^3-5
	C^3-2	73.60	4.04 (t, 8.4)	C^3-1, C^3-3	
	C^3-3	86.74	4.29 (t, 8.6)	C^3-4, C^3-2	H^3-1
	C^3-4	69.20	3.99 (t, 8.7)	C^3-5, C^3-6	
	C^3-5	77.11	3.98 (m)		H^3-1
	C^3-6	61.45	4.36 (br. d, 11.1)		
			4.11 (dd, 5.2, 12.0)		
3MeGlc (4)	C^4-1	104.56	5.30 (d, 7.6)	C^3-3	H^3-3, H^4-3, H^4-5
	C^4-2	74.59	3.97 (t, 8.3)	C^4-1, C^4-3	
	C^4-3	86.99	3.79 (t, 8.7)	OMe, C^4-2, C^4-4	H^4-1
	C^4-4	70.39	3.99 (t, 9.4)		
	C^4-5	77.55	4.04 (m)		H^4-1
	C^4-6	61.75	4.16 (m), 4.49 (m)		
	OMe	60.79	3.92 (s)	C^4-3	
Xyl (5)	C^5-1	105.30	5.32 (d, 7.1)	C^2-2	H^2-2, H^5-3, H^5-5
	C^5-2	74.76	4.07 (t, 7.7)	C^5-1, C^5-3	
	C^5-3	76.44	4.17 (t, 8.6)	C^5-4, C^5-2	H^5-1
	C^5-4	70.15	4.22 (m)	C^5-3	
	C^5-5	66.39	4.42 (dd, 5.0, 11.5)	C^5-1, C^5-4, C^5-3	
			3.71 (dd, 9.0, 11.4)	C^5-1, C^5-4, C^5-3	H^5-1

H]$^+$ at *m*/*z* 1137 in MALDI-TOF MS corresponds to the loss of sulfate group, and a peak of a fragment ion at *m*/*z* 1017 in MALDI-TOF(–) MS, to the loss of the 3-*O*-methylglucose terminal residue.

Therefore, okhotoside A_1-1 (**I**) is 16β-acetoxy-3-β-hydroxy-3-*O*-[3-*O*-methyl-β-*D*-glucopyranosyl-(1⟶3)-β-*D*-xylopyranosyl-(1⟶4)-β-*D*-quinovopyranosyl-(1⟶2)-4-*O*-sodiumsulfonyl-β-*D*-xylopyranosyl]-holost-7-en-23-one.

Five signals of anomeric carbon atoms observed at δ 101.43–105.28 ppm in the ^{13}C NMR spectrum of okhotoside A_2-1 (**II**) are related to its carbohydrate fragment. They were correlated using an HSQC spectrum with five signals of the corresponding protons at 4.84 (d, *J* 7.3 Hz), 5.52 (d, *J* 7.7 Hz), 5.08 (d, *J* 8.0 Hz), 5.30 (d, *J* 7.6 Hz), and 5.32 ppm (d, *J* 7.1 Hz); this proved the presence of five monosaccharide residues in the carbohydrate chain of (**II**). The spin-coupling values of anomeric protons indicate the presence of β-glycoside bonds (Table 3 [16]).

A comparison of a portion of ^{13}C NMR spectrum corresponding to carbohydrate chain of okhotosidea A_2-2 with the corresponding portion of spectrum of monosulfated pentaoside cucumarioside A_2-2, earlier isolated from holothurian *C. japonica* [19], showed the

coincidence of signals of four monosaccharide residues of these compounds. The signals of only one residue, quinovose, which occupies position 2 in the carbohydrate chain of cucumarioside A_2-2, did not correspond to the signals in the spectrum of okhotoside A_2-1 (**II**): the ^{13}C NMR signals in the area of $\delta \sim 18$ ppm in the spectrum of cucumarioside A_2-2 and the signals in the area of $\delta \sim 1.7$ ppm in the spectrum of okhotoside A_2-1 (**II**) did not coincide. No ^1H NMR signals characteristic of methyl groups of 6-deoxyhexoses (i.e., the signals of methyl group of the quinovose residue, which is present in the majority of carbohydrate chains of holothurian triterpenoid glycosides) were also exhibited by okhotoside A_2-1 (**II**). At the same time, we observed an additional signal at δ 61.06 (CH_2) ppm in ^{13}C NMR spectrum of glycoside (**II**) in comparison with the spectrum cucumarioside A_2-2; it is characteristic of C6 atoms of the residues of glucose and 3-*O*-methylglucose, monosaccharides typical of holothurian glycosides. This information allowed us to suggest the presence of a glucose residue in position 2 of the carbohydrate chain of okhotoside A_2-1 (**II**) instead of a quinovose residue in the carbohydrate chain of cucumarioside A_2-2 [19].

An analysis of 1D-TOCSY and COSY spectra of glycoside (**II**) allowed us to ascribe the proton signals in the isolated spin systems of all the monosaccharide residues. In turn, they were correlated by HSQC spectrum with the signals of the corresponding carbon atoms. The sequences of monosaccharide residues in the carbohydrate chains of okhotoside A_2-1 (**II**) were determined using the cross peaks in the HMBC spectrum between: H1 of the xylose residue (the first monosaccharide residue) and C3 of aglycone, H1 of the glucose residue (the second monosaccharide residue) and C2 of the xylose residue (the first monosaccharide residue), H1 of the glucose residue (the third monosaccharide residue) and C4 of glucose (the second monosaccharide the residue), H1 of the terminal 3-*O*-methylglucose (the fourth monosaccharide the residue) and C3 of the glucose residue of (the third monosaccharide residue), and H1 of the terminal xylose residue (the fifth monosaccharide residue) and C2 of glucose (the second monosaccharide residue). The structure of the pentasaccharide carbohydrate chain of okhotoside A_2-1 (**II**) was also confirmed by NOESY spectra (Table 3).

A part of ^{13}C NMR spectrum of okhotoside A_2-1 (**II**) related to aglycone exhibits the signals of holostane aglycone: the signal of quaternary carbon atom C18 at 180.25 ppm, the signals at 120.23 (C7) and 145.59 (C8) ppm of carbons included in the 7(8) double bond of nucleus, the signals at 145.43 (C25) and 110.9 (C26) ppm related to the terminal 25(26) double bond of the side chain, and *O*-acetyl group at C16 (signals at 75.29 (C16), 170.76 ($O\underline{C}OCH_3$) and 21.30 ppm ($O\mathbf{C}O\underline{C}H_3$) (Table 4). Thus, the aglycone of okhotoside A_2-1 (**II**) differs from the okhotoside A_1-1 (**I**) aglycone by the structure of side chain in which keto group at C23 was absent and a terminal double bond was present.

The empirical formula of okhotoside A_2-1 (**II**) was determined as $C_{61}H_{95}O_{31}SNa$ on the basis of ^{13}C NMR spectrum and the peaks of pseudo-molecular ions $[M_{Na} + Na]^+$at *m/z* 1401.5 and $[M_{Na} + K]^+$ at *m/z* 1417.5 in MALDI TOF(+) MS, in which also the peaks of fragment ions $[M_{Na} + Na - SO_3Na + H]^+$ at *m/z* 1299.4 due to the cleavage of sulfate group and at *m/z* 1225.4 due to the cleavage of the terminal residue of 3-*O*-methylglucose were present.

All the data proved that okhotoside A_2-1 (**II**) is 16β-acetoxy-3β-hydroxy-3-*O*-{3-*O*-methyl-β-*D*-glucopyranosyl-(1⟶3)-β-*D*-glucopyranosyl-(1⟶4)-[β-*D*-xylopyranosyl-(1⟶2)]-β-*D*-glucopyranosyl-(1⟶2)-4-*O*-sodiumoxysulfonyl-β-*D*-xylopyranosyl}-holosta-7,25-diene.

The aglycone with such a structure is rather widely distributed in holothurian glycosides, because other four glycosides containing identical aglycones are known: cucumarioside A_0-2 from holothurian *C. japonica* [17, 18], neothyonidioside C from holothurian *Neothyonidium magnum* [20], and thyonidiosides A and B from holothurian *Thyone aurea* [21]. At the same time, the carbohydrate chain of okhotoside A_2-1 (**II**) is new and earlier has not been found in holothurian glycosides and, moreover, no glycosides with a glucose residue in position 2 of carbohydrate chain have previously been isolated from holothurians of the genus *Cucumaria*.

A comparison of ^{13}C NMR spectrum of (**III**) with the spectrum of cucumarioside A_0-1, earlier isolated from *C. japonica* [18], allowed us to draw a conclusion on the identity of their structures. Thus, new okhotoside A_1-1 (**I**) differs from cucumarioside A_0-1 (**III**) by the absence of only terminal xylose residue. This was confirmed by a comparison of ^{13}C NMR spectra of these compounds. The signals of an additional xylose residue in the ^{13}C NMR spectrum of cucumarioside A_0-1 (**III**) in comparison with the spectrum of okhotoside A_1-1 (**I**). The position of this residue at C2 of the quinovose residue was proved by a downfield shift of C2 signal of quinovose by 5.83 ppm in comparison with the C2 signal of quinovose in the spectrum of (**I**) and an upfield shift of the C3 signal of the same residue by 0.26 ppm due to the glycosylation effects. Glycoside (**III**) was identified as 16β-aceoxy-3β-hydroxy-3-*O*-{3-*O*-methyl-β-*D*-glucopyranosyl-(1⟶3)-β-*D*-xylopyranosyl-(1⟶4)-[β-*D*-xylopyranosyl-(1⟶2)]-β-*D*-quinovopyranosyl-(1⟶2)-4-*O*-sodiumoxysulfonyl-β-*D*-xylopyranosyl}-holost-7-en-23-one, like the earlier isolated cucumarioside A_0-1[18], by a comparison of ^{13}C NMR spectra of these compounds and the identification of their physical constants.

Our results showed that the structures of holothurian glycosides of the various species belonging to the genus *Cucumaria*, on the one hand, differ from each other, i.e., possess the species specificity, and on the other hand, are characterized by a number of the com-

Table 4. NMR spectra of the aglycone part of okhotoside A_2-1 (**II**) in 4 : 1 C_5D_5N–D_2O

Aglycone atom number	δ_C, DEPT	δ_H (multiplicity, *J*, Hz)	HMBC	NOESY
1	35.90	1.40 (m)		**H**: 5, 11, 19
2	26.70	1.95 (m), 2.03 (m)		
3	89.22	3.31 (dd, 4.2, 11.6)		**H**: 5, 31, H^1-1
4	39.43			
5	47.80	1.00 (dd, 5.0, 10.0)	**C**: 4, 10, 19, 30	**H**: 1, 3
6	23.08	2.00 (m)		**H**: 30, 31
7	120.23	5.69 (m)		**H**: 15, 32
8	145.59			
9	47.01	3.40 (br. d, 14.0)		**H**: 19
10	35.34			
11	22.45	1.59 (m), 1.81 (m)		**H**: 1
12	31.24	2.21 (m)		**H**: 17, 21
13	59.31			
14	47.29			
15	43.52	1.71 (m)		
		2.66 (dd, 7.4, 12.2)		**H**: 7
16	75.29	5.93 (br. q, 8.7)		**H**: 32
17	54.46	2.78 (d, 9.0)	**C**: 12, 13, 18, 21	**H**: 32, 21, 12
18	180.25			
19	23.80	1.16 (s)	**C**: 1, 5, 9, 10	**H**: 1, 9
20	85.58			
21	28.09	1.62 (s)	**C**: 17, 20, 22	**H**: 17, 22
22	38.27	2.35 (m), 1.91 (m)		
23	22.90	1.56 (m), 1.44 (m)		
24	38.18	2.01 (m)		
25	145.43			
26	110.87	4.83 (br. s)	**C**: 24, 27	
		4.84 (br. s)		
27	22.08	1.75 (s)	**C**: 24, 25, 26	
30	17.27	1.15 (s)	**C**: 3, 4, 5, 31	**H**: 6
31	28.57	1.28 (s)	**C**: 3, 4, 5, 30	**H**: 3, 6
32	32.20	1.27 (s)	**C**: 8, 13, 14, 15	**H**: 7, 16, 17
$OCO\underline{C}H_3$	21.30	2.12 (s)		
$O\underline{C}OCH_3$	170.76			

mon features, such as the presence of groups of mono-, di-, and trisulfated glycosides and pentaoside carbohydrate chains branched at the residue in position 2 of carbohydrate chain, which allow the classification of a species to the genus [4, 5]. The holothurian *C. okhotensis* [10] and the majority of other species of the genus *Cucumaria* contains a complex mixture of triterpenoid glycosides of various groups. Okhotosides A_1-1 (**I**) and A_2-1 (**II**) are new compounds, with glycoside (**II**) being characterized by the presence of a carbohydrate chain with a glucose residue in position 2. Such structures had not been found earlier among the holothurians of the *Cucumaria* genus. This fact indicates an essential structural difference of the monosulfated glycosides of *C. okhotensis* from the glycosides of similar groups in other species of the genus *Cucumaria* and also allows us to speak already at this stage of work on the species specificity of *C. okhotensis* glycosides and confirm the validity of the establishment of a new species *C. okhotensis* within the genus *Cucumaria*.

At the same time, the carbohydrate chain of the new okhotoside A_1-1 (**I**) is identical to the carbohydrate

chain of frondoside A_1, earlier isolated from *C. frondosa* [15], whereas the carbohydrate chain of cucumarioside A_0-1 (**III**) is identical to the chain of frondoside A [22]. Thus, the glycoside pairs okhotoside A_1-1 (**I**)–cucumarioside A_0-1 (**III**) from *C. okhotensis* and frondoside A_1–frondoside A from *C. frondosa* can be considered as structural analogues confirming the belonging of the holothurian *C. okhotensis* to the genus *Cucumaria*. Moreover, okhotoside A_1-1 (**I**) is obviously a biosynthetic precursor of cucumarioside A_0-1 (**III**).

This information imposes rigid requirements on the standardization of raw materials used for the manufacturing of drugs and biologically active additives to food from the so-called *Cucumaria japonica* Therefore, the practical use of glycoside fractions from different Far-East cucumaria requires their careful analysis, because the properties of preparations could change along with variations in their glycoside composition.

EXPERIMENTAL

Biological material. The holothurian *C. okhotensis* was collected at trade dredging near the western coast of Kamchatka in autumn of 2001 from the depth of 28 m. The species attribution was achieved by Dr. Sci. (Biol.) V.S. Levin.

Instruments and materials. Melting points of glycosides were determined with the help of a Kofler-Thermogenerate apparatus. Optical rotation was measured on a Perkin-Elmer 343 polarimeter. Spectra of ^{13}C and ^{1}H NMR were obtained on spectrometers Bruker AMX 500 and Bruker DPX 300. Chemical shifts in NMR spectra are given in δ scale in ppm relative to tetramethylsilane as internal standard ($\delta_{TMS} = 0$). MALDI TOF mass spectra were measured on a BIFLEX III (Bruker) mass spectrometer with a pulse extraction of ions. α-Cyano-4-hydroxycinnamic acid was used as a matrix. An Agilent 1100 chromatograph supplied with a differential refractometer was used for HPLC. A teflon powder Polychrom-1 (NPO Biolar, Latvia) was used for hydrophobic column chromatography, with buffer A being a solution of $Na_2HPO_4 \cdot 12H_2O$ (540 mg) in water (100 ml).

Isolation of triterpene glycosides. A ground holothurian was extracted with hot ethanol at reflux; the mass of dry residue after the extraction was 1385 g. The extract was decanted and evaporated in a vacuum. The residue was dissolved in water and chromatographed on a Polychrom-1 column. The column was packed in ethanol, washed with water, substances were loaded in water, and washed with water before negative reaction to chloride anion with silver nitrate. The mixture of glycosides was eluted from the column with 50% ethanol. The glycoside fraction was subjected to repeated column chromatography on silica gel in systems chloroform–ethanol–water 100 : 75 : 10, 100 : 100 : 17, and 100 : 125 : 25 that provided the separation to subfractions of mono-, di-, and trisulfated compounds. The separation of the monosulfated glycosides to groups A, A_1, and A_2 was monitored by TLC, using the glycosides of similar groups from the holothurian *C. frondosa* as standards. The subfractions of monosulfated okhotosides of groups A_1 (74 mg) and A (130 mg) were additionally purified on a Sephadex LH-20 column eluted with 40% ethanol, which results in the okhotoside subfractions of groups A_1 (45 mg) and A (85 mg). The subfraction of the okhotoside group A_2 (94 mg) was obtained after the repeated column chromatography on silica gel.

The okhotoside subfraction of group A_1 (45 mg) was separated with the help of HPLC on a Diasphere-110 C-18 column (4 × 250 mm) eluted with 60 : 38 : 2 MeOH–H_2O–buffer A mixture. The resulting individual compounds were desalted on Polychrom-1 as described above. We obtained 6 mg of individual glycoside (**I**). The HPLC separation of the subfraction of okhotosides of group A_2 (94 mg) on a Diasphere-110 C-18 column eluted with 64 : 33 : 3 MeOH–H_2O–buffer A mixture led to 6 mg (after desalting on a Polychrom-1 column as described above) of individual glycoside (**II**). The subfraction of okhotosides A (85 mg) was separated with the help of successive HPLC on columns Zorbax ODS (10 × 250 mm) eluted with 50 : 48 : 2 EtOH–H_2O–buffer A and Diasphere-110 C-18 column eluted with 60 : 38 : 2 MeOH–H_2O–buffer A. We obtained 10 mg (after desalting on a Polychrome-1 column as described above) of individual (**III**).

Okhotoside A_1-1 (I); mp 268–269°C, $[\alpha]_D^{20}$ –8° (*c* 0.1, pyridine); MALDI TOF MS, *m/z* 1239 [M_{Na} + Na]$^+$ (empirical formula $C_{55}H_{85}O_{26}SNa$), 1137 [M_{Na} + Na – SO_3Na + H]$^+$, 1193 [M_{Na} – Na]$^-$, 1017 [M_{Na} – Na – 3MeGlc + H]. For NMR spectra, see Tables 1, 2.

Okhotoside A_2-1 (II); mp 209–210°C, $[\alpha]_D^{20}$ –19° (*c* 0.1, pyridine); MALDI TOF MS, *m/z* 1401.5 [M_{Na} + Na]$^+$ (empirical formula $C_{61}H_{95}O_{31}SNa$), 1417.5 [M_{Na} + K]$^+$, 1299.4 [M_{Na} + Na – SO_3Na + H]$^+$, 1225.4 [M_{Na} + Na – 3MeGlc + H]$^+$, 1123.4 [M_{Na} + Na – SO_3Na – 3MeGlc + H]$^+$, 1123.4 [M_N + Na – SO_3Na – 3MeGlc + H]$^+$, 1355.4 [M_{Na} – Na]$^-$, 1179.4 [M_{Na} – Na – 3MeGlc]$^-$. For NMR spectra, see Tables 3, 4.

Cucumarioside A_0-1 (III); mp 220–222°C, $[\alpha]_D^{20}$ –12° (*c* 0.1, pyridine), for NMR spectrum, see Table 5.

Determination of absolute configuration of monosaccharides. Acidic hydrolysis of okhotoside A_1-1 (**I**) (3 mg) was carried out in 0.2 M TFA (0.3 ml) in a sealed ampoule on a water bath at 100°C for 30 min. The precipitated aglycone was extracted with chloroform, and a water solution of sugars was evaporated in a vacuum. One drop of TFA and 0.2 ml of (–)-2-octanol (Aldrich) were added to the dry residue, sealed in an ampoule, and heated on a glycerol bath at 130°C on a magnetic stirrer for 6 h. The mixture was evaporated in a vacuum and treated with a pyridine–

Table 5. ^{13}C NMR spectrum of cucumarioside A_0-1 (**III**) in C_5D_5N

Aglycone atom number	δ_C, DEPT	Residue	Atom	δ_C, DEPT
1	35.88	Xyl (1)	C^1-1	104.91
2	26.82		C^1-2	81.62
3	89.04		C^1-3	76.43
4	39.44		C^1-4	75.30
5	47.71		C^1-5	64.26
6	23.18			
7	120.32	Qui (2)	C^2-1	102.49
8	145.43		C^2-2	82.00
9	46.94		C^2-3	74.92
10	35.41		C^2-4	85.18
11	22.35		C^2-5	71.10
12	30.95		C^2-6	17.80
13	57.62			
14	47.43	Xyl (3)	C^3-1	104.72
15	43.53		C^3-2	73.29
16	75.61		C^3-3	87.17
17	55.02		C^3-4	68.93
18	179.22		C^3-5	66.38
19	23.81			
20	82.03	3MeGlc (4)	C^4-1	105.54
21	29.44		C^4-2	74.92
22	52.64		C^4-3	87.88
23	207.64		C^4-4	70.44
24	51.40		C^4-5	78.16
25	24.22		C^4-6	61.97
26	22.36		OMe	60.64
27	22.17			
30	17.29	Xyl (5)	C^5-1	105.28
31	28.55		C^5-2	75.29
32	31.98		C^5-3	76.10
$OCO\underline{C}H_3$	21.10		C^5-4	70.26
$O\underline{C}OCH_3$	169.02		C^5-5	66.40

acetic anhydride mixture (1 : 1, 0.6 ml) for 24 h at room temperature. The acetylated derivatives of (–)-2-octyl glycosides were analyzed by GC in the presence of standards of the corresponding monosaccharides. The temperature regime: 100°C (0.5 min) – 5°N/min gradient – 250°C (10 min), injector temperature 150°C, the detector temperature 280°C. The peaks with the following retention times were identified at the GC analysis of (**I**): (I) 24.48, 24.73, and 25.02 min (*D*-xylose); 23.02, 24.18, and 24.60 min (*D*-quinovose); and 28.23, 28.55, 28.85, and 29.09 min (3-*O*-methyl-*D*-glucose). The retention times of monosaccharide standards in the mixture were: 24.48, 24.73, and 25.02 min (*D*-xylose); 23.02, 24.18, and 24.60 mines (*D*-quinovose); and 28.23, 28.55, 28.85, and 29.09 min (3-*O*-iaoee-*D*-glucose). The peaks for *L*-isomers of monosaccharides were identified at: 24.24, 24.30, 24.83, and 27.97 min (*L*-xylose) and 27.41 and 27.86 min (3-*O*-iaoee-*L*-glucose). The retention times of *L*-isomers were determined for the "virtual derivatives" of L-series, (+)-2-octyl glycosides corresponding to the sugars of the *D*-series [22].

ACKNOWLEDGMENTS

We thank Dr. Sci. (Chem.) E.V. Evtushenko for the kind gift of monosaccharide standards.

This work was supported by the grant of the President of the Russian Federation for support of leading scientific schools, program of the Presidium of the Russian Academy of Science Molecular and Cellular Biology, the Russian Fund for Basic Research, project no. 06-04-96016, Far-East Division of the Russian Academy of Sciences grant nos. 06-III-B-05-128 and 06-III-B-05-126, Far-East and Urals Divisions of the Russian Academy of Sciences grant no. 06-2U-0-05-009, and NATO LST.NR.CLG. 981098.

REFERENCES

1. Kalinin, V.I, Anisimov, M.M., Prokofieva, N.G., Avilov, S.A., Afiyatullov, Sh.Sh., and Stonik, V.A., in *Echinoderm Studies,* Jangoux, M. and Lawrence, J.M.A.A., Eds., Balkema, Rotterdam: Brookfield, 1966, pp. 139–184.
2. Elyakov, G.B., Stonik, V.A., Afiyatullov, Sh.Sh., Kalinovskii, A.I., Sharypov, V.F., and Korotkikh, L.Ya., *Dokl. Akad. Nauk SSSR*, 1981, vol. 259, pp. 1367–1369.
3. Chludil, H.D., Murray, A.P., Seldes, A.M., and Maier, M.S., *Studies Nat. Prod. Chem.*, 2003, vol. 28, pp. 587–615.
4. Avilov, S.A., Kalinin, V.I., and Smirnov, A.V., *Biochem. Syst. Ecol.*, 2004, vol. 32, pp. 715–733.
5. Stonik, V.A., Kalinin, V.I., and Avilov, S.A., *J. Nat. Toxins*, 1999, vol. 8, pp. 235–248.
6. Levin, V.S. and Gudimova, E.N., *Zool. Zh.,* 1997, vol. 76, pp. 575–584.
7. Baranova, Z.I., in *Novoe v sistematike morskikh bespozvonochnykh* (News in Systematics of Marine Invertebtata), Leningrad: ZIN AN SSSR, 1980, pp 109–120.
8. Levin, V.S. and Stepanov, V.G., *Biol. Morya,* 2002, vol. 28, pp. 66–69.
9. Stepanov, V.G. and Pingal'chuk, O.A., *Zool. Zh.,* 2002, vol. 81, pp. 1392–1397.
10. Levin, V.S., *Biol. Morya,* 2003, vol. 29, pp. 202–205.
11. Avilov, S.A., Antonov, A.S., Silchenko, A.S., Kalinin, V.I., Kalinovsky, A.I., Dmitrenok, P.S., Stonik, V.A., Riguerpa, R., and Jimenes, C., *J. Nat. Prod.*, 2003, vol. 66, pp. 910–916.
12. Grishin, Yu.I., Kovalevskaya, A.M., Stonik, V.A., Avilov, S.A., Vlaznev, V.P., and Slugin, V.S., *Novosti Zverovodstva,* 1991, no. 2, pp. 19–23.

13. Grishin, Yu.I., Kovalevskaya, A.M., Stonik, V.A., Avilov, S.A., Vlaznev, V.P., and Slugin, V.S., RF Patent 2 036 654, Byull. Izobret., No. 16, 1995.
14. Avilov, S.A., Kalinin, V.I., Silchenko, A.S., Aminin, D.L., Agafonova, I.G., Stonik, V.A., Woodward, C., and Collin, P.D., *Process for isolating sea cucumber saponin frondoside A, and immunomodulatory methods of use*, USPTO, nos. 60/579,355, priority from June 14, 2004, PCT/RU 2005/000306.
15. Avilov, S.A., Kalinin, V.I., Drozdova, O., Kalinovskii, A.I., Stonik, V.A., and Gudimova, E.N., *Khim. Prir.. Soedin.*, 1993, no. 2, pp. 260–263.
16. Shashkov, A.S. and Chizhov, O.S., *Bioorg. Khim.,* 1976, vol. 2, pp. 437–497.
17. Drozdova, O.A., Avilov, S.A., Kalinovskii, A.I., and Stonik, V.A., *Khim. Prir.. Soedin.*, 1992, no. 5, pp. 590–591.
18. Drozdova, O.A., Avilov, S.A., Kalinovskii, A.I., and Stonik, V.A, *Khim. Prir.. Soedin.*, 1992, no. 5, p. 593.
19. Avilov, S.A., Tishchenko, L.Ya., and Stonik, V.A., *Khim. Prir. Soedin.*, 1984, no. 6, pp. 799–800.
20. Avilov, S.A., Kalinovskii, A.I., and Stonik, V.A., *Khim. Prir. Soedin.*, 1990, no. 1, pp. 53–57.
21. Bonnard, I. and Rinehart, K.L., *Tetrahedron,* 2004, vol. 60, pp. 2987–2992.
22. Leontein, K., Lindberg, B., and Lönngren, J., *Carbohydr. Res.,* 1978, vol. 62, pp. 359–362.